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# ProcartaPlex®

## Multiplex Immunoassay

Using Magnetic Beads
For Serum, Plasma, and Cell Culture Supernatant Samples

Instructions for
Rat Isotyping Assays


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## Intended Use

This user manual is for a ProcartaPlex® Rat Antibody Isotyping Panel from the eBioscience division of Affymetrix to perform quantitative, multiplexed protein measurements from serum, plasma, and cell culture supernatant samples using magnetic beads technology from Luminex®. Other biological samples might be suitable for use in the assay.

**NOTE: For the most current version of user documentation, go to our website at www.ebioscience.com**

**NOTE: ProcartaPlex Rat Antibody Isotyping Panel cannot be combined with other ProcartaPlex pre-mixed Panels and Simplex Kits.**

## How it Works

ProcartaPlex Immunoassays incorporate magnetic microsphere technology licensed from the Luminex Corporation to enable the simultaneous detection and quantitation of multiple protein targets in diverse matrices. The platform allows the simultaneous detection from a single sample of up to 100 protein targets on the Luminex 200/100 and FLEXMAP 3D® platforms and 50 protein targets on the MAGPIX® platform.

## Materials Provided and Storage Conditions

ProcartaPlex Immunoassay Kits contain the components listed below. Refer to the Certificate of Analysis for quantities and details of components supplied. Expiration date is stated on the kit when stored between 2-8°C. Do not use past kit expiration date.

| Components Supplied |
|---|
| Antigen Standards, premixed |
| Detection Antibody, premixed (50X) [1] |
| Antibody Magnetic Beads, premixed[1] |
| Wash Buffer Concentrate (10X)[1] |
| Detection Antibody Diluent[1] |
| Universal Assay Buffer Concentrate (10X)[1] |
| Reading Buffer[1] |
| PCR 8-Tube Strip |
| 96-Well Flat Bottom Plate |
| Black Microplate Lid |
| Plate Seals |

[1] Contains sodium azide. See WARNING.

**WARNING: All chemicals should be considered potentially hazardous. We recommend that this product and its components be handled by those trained in laboratory techniques and be used according to the principles of good laboratory practice. This kit contains small quantities of sodium azide. Sodium azide is highly toxic and reactive in the pure form. At this product's concentration, though not classified as hazardous, build up of sodium azide may react with lead and copper plumbing to form highly reactive explosive metal azide. Dispose of the product in accordance with all State and local regulations.**

## Precautions and Technical Hints

- Thoroughly read this user manual and Certificate of Analysis that is included with the assay kit. The product insert may contain specific instructions for proper use of your kit.
- For Luminex 100/200 and FLEXMAP 3D instruments initiate the startup protocol to warm up the lasers for at least 30 minutes. Ensure that the Luminex machine is calibrated according to the manufacturer's instructions. MAGPIX instrument doesn't require additional warm up.
- When working with samples and standards, change the pipette tips after every transfer and avoid creating bubbles when pipetting.
- During the incubation steps, cover the 96-Well Flat Bottom Plate with the Black Microplate Lid provided in the kit to minimize exposure of the beads to light.
- Be careful not to invert the 96-Well Flat Bottom Plate during the assay or allow contents from one well to mix with another well.
- Use a multi-channel pipette and reagent reservoirs whenever possible to achieve optimal assay precision.
- Store the reconstituted standards (including standard diluent sets) on ice before adding to the 96-Well Flat Bottom Plate.

## Required Equipment and Materials Not Supplied

- MAGPIX, Luminex® 100/200, FLEXMAP 3D, or Luminex-based Instrument.
- Glass-distilled or deionized water.
- Adjustable single and multi channel pipettes with disposable tips.
- Multichannel pipette reservoir.
- Beakers, flasks, cylinders necessary for preparation of reagents.
- Hand-Held Magnetic Plate Washer, Vortex mixer and Microtiter plate shaker.

## Sample Preparation

- For frozen samples, thaw samples on ice and mix well by vortexing followed by centrifugation to remove particulates. Avoid multiple freeze/thaw cycles.
- If there is a high lipid content in the sample, centrifuge at 10,000 x *g* for 10 min at 2-8 °C and transfer contents to a new tube.

For preparation instruction of tissue homogenates and lysates from cultured cells refer to **www.ebioscience.com/resources/best-protocols/multiplexing.htm**

### Plasma Sample Preparation

| | Action |
|---|---|
| **A.** | Collect samples in sodium citrate or EDTA tubes. When using heparin as an anticoagulant, no more than 10 IU of heparin per mL of blood collected should be used since an excess of heparin may give falsely high values of some of the analytes. |
| **B.** | Centrifuge samples at 1,000 x *g* at 4 °C for 10 min within 30 min of blood collection. |
| **C.** | Collect the plasma fraction. |
| **D.** | Use immediately or aliquot and store at -80 °C. |

### Serum Sample Preparation

We recommend to spin down serum samples at 1000 x *g* for 10 min at 20-25 °C before running the assay.

| Action |
|---|
| **A.** Allow blood to clot for 20-30 min at 20-25 °C. |
| **B.** Centrifuge at 1,000 x *g* for 10 min at 20-25 °C. |
| **C.** Collect the serum fraction. (Alternatively, use any standard serum separator tube following the manufacturer's instructions.) |
| **D.** Use immediately or aliquot and store at -80 °C. |

### Diluting Samples with High Concentration of Target Analytes

The analytes included in the kit have high serum or plasma concentrations.

1:40.000-fold dilution in Universal Assay Buffer (1X) is appropriate for most serum and plasma samples. For samples that yield results outside the range of the standard curve, a lower or higher dilution might be required. or cell culture supernatant, a 1:200 dilution in cell culture medium is suggested.

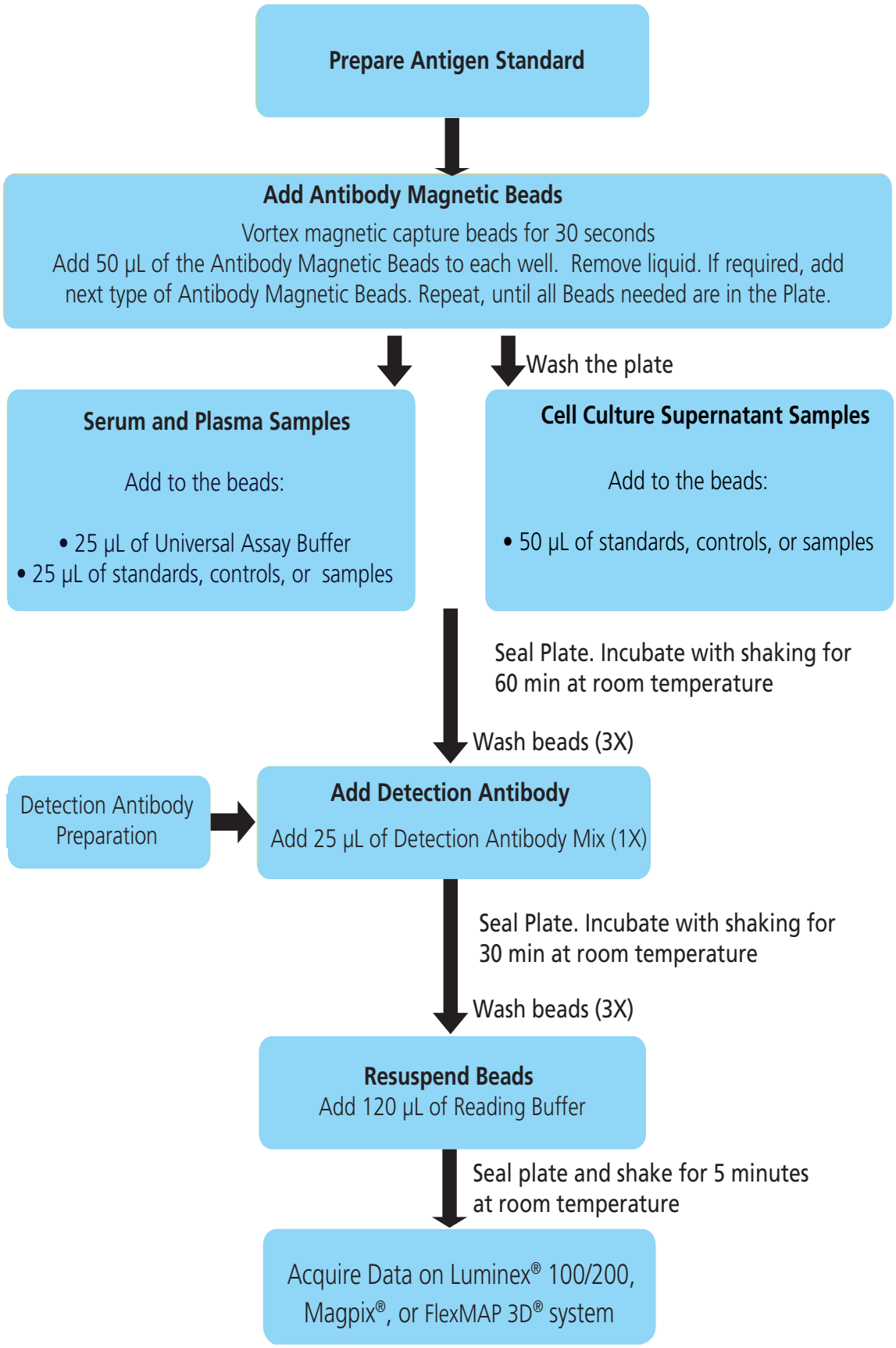
Each serum or plasma sample has to be diluted 1:40000 according to following scheme:

| 1:200 Dilution | Sample Volume | UAB Volume |
|---|---|---|
| Dilution 1 (1:200) | 10 µL | 1990 µL |
| Dilution 2 (1:200) | 10 µL | 1990 µL |

If the samples are taken from cell culture supernatant prepare a 1:200 dilution:

| 1:200 Dilution | Sample Volume | CCM Volume |
|---|---|---|
| Dilution (1:200) | 10 µL | 1990 µL |

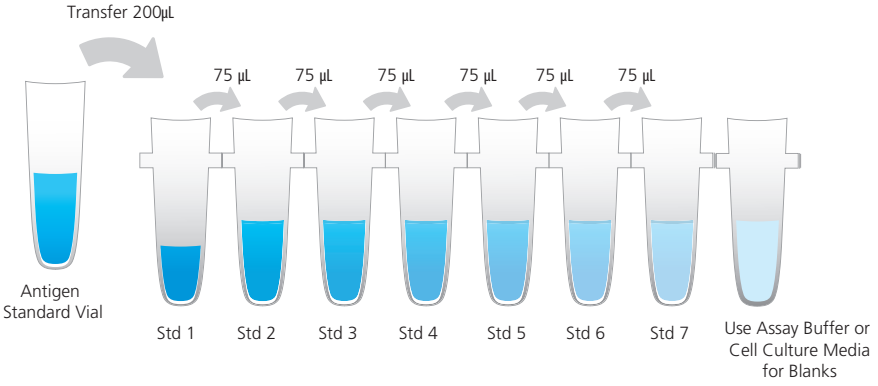
## Assay Protocol Overview


Prepare Antigen Standard
Add Antibody Magnetic Beads
Vortex magnetic capture beads for 30 seconds
Add 50 µL of the Antibody Magnetic Beads to each well. Remove liquid. If required, add next type of Antibody Magnetic Beads. Repeat, until all Beads needed are in the Plate.
Wash the plate
Serum and Plasma Samples
Add to the beads:
• 25 µL of Universal Assay Buffer
• 25 µL of standards, controls, or samples
Cell Culture Supernatant Samples
Add to the beads:
• 50 µL of standards, controls, or samples
Seal Plate. Incubate with shaking for 60 min at room temperature
Wash beads (3X)
Detection Antibody Preparation
Add Detection Antibody
Add 25 µL of Detection Antibody Mix (1X)
Seal Plate. Incubate with shaking for 30 min at room temperature
Wash beads (3X)
Resuspend Beads
Add 120 µL of Reading Buffer
Seal plate and shake for 5 minutes at room temperature
Acquire Data on Luminex® 100/200, Magpix®, or FlexMAP 3D® system

## Preparation of Reagents

### Antigen Standard

Carefully review the Certificate of Analysis for kit specific Antigen Standard preparation instructions. Each kit is shipped with two identical vials of each premixed antigen standard set from the same lot to permit the user to run the assay twice if running a partial plate.

| Step | Action |
|---|---|
| **Step 1. Reconstitution and pooling of Standards** | **A.** Centrifuge the antigen standard set vial at 2000 x *g* for 10 sec.<br>**B.** Add 250 µL of sample type specific buffer into the standard set vial. For serum or plasma samples, use Universal Assay Buffer and for cell culture supernatant samples use the cell culture media that was used to culture the cells.<br>**C.** Gently vortex the vial for 30 seconds and centrifuge at 2000 x *g* for 10 seconds to collect contents at the bottom of the vial.<br>**D.** Incubate on ice for 10 min to ensure complete reconstitution. |
| **Step 2. Prepare 3-Fold Serial Dilution** | **A.** Refer to Certificate of Analysis for the value of each premixed standard with assigning S1 values for each analyte for the current lot.<br>**B.** Prepare a 3-fold serial dilution of the reconstituted standard(s) using the PCR 8-tube strip provided. Label tubes Std1, Std2, Std3, Std4, Std5, Std6 and Std7.<br>**C.** Add 200 µL of the reconstituted antigen standards into the first tube of the strip tube and label as Standard 1 (Std1).<br>**D.** Add 150 µL of sample type specific standard buffer into Std tubes 2-7. Use Universal Assay Buffer for serum or plasma samples and cell culture media for culture supernatant samples.<br>**E.** Transfer 75 µL of the reconstituted antigen standards from Tube 1 into Tube 2.<br>**F.** Mix by pipetting up and down for a total of 10 times.<br>**G.** Change the pipette tip and transfer 75 µL of the mixed standards from Tube 2 into Tube 3.<br>**H.** Mix by pipette up and down 10 times.<br>**I.** Repeat steps E-H for Std tubes 4-7.<br>**J.** Add 200 µL of Universal Assay Buffer or cell culture media into tube 8 which serves as a blank. Keep on ice until ready to use.<br>  |

## Assay Protocol

| Step | Action |
|---|---|
| **Step 1. Prepare 1X Wash Buffer** | Bring the Wash Buffer Concentrate (10X) to room temperature and vortex for 15 seconds. Mix 20 mL of the Wash Buffer Concentrate (10X) with 180 mL dd$H_2O$. Wash Buffer (1X) can be stored at 2-8 °C for up to 6 months. |
| **Step 2. Define the plate map** | Mark the standard, sample and blank wells using the plate map at the end of this manual. |
| **Step 3a. Add the Antibody Magnetic Beads** | **A.** Vortex the Antibody Magnetic Beads for 30 sec.<br>**B.** Add 50 µL of the Antibody Magnetic Beads to each well. Use a multi-channel pipette for this step as well as the steps below. |
| **Step 4. Wash Antibody Magnetic Beads** | **A.** Securely insert the 96-Well Flat Bottom Plate into the Hand-Held Magnetic Plate Washer and ensure that the plate is held in place by the tabs and wait 2 min to allow the Antibody Magnetic Beads to accumulate on the bottom of each well.<br>**B.** Remove the liquid in the wells by quickly inverting the Hand-Held Magnetic Plate Washer and 96-Well Flat Bottom Plate assembly over a sink or waste container. Do not remove the 96-Well Flat Bottom Plate from the Hand-Held Magnetic Plate Washer. Blot the inverted assembly onto several layers of paper towels or absorbent surface to remove any residual solution.<br>**C.** Add 150 µL of Wash Buffer (1X) into each well and wait 30 seconds to allow the beads to accumulate on the bottom of each well.<br>**D.** Remove the Wash Buffer in the wells by quickly inverting the plate and then blotting onto an absorbent towel to remove any residual solution.<br>**E.** Remove the 96-Well Flat Bottom Plate from the Hand Held Magnetic Plate Washer and proceed to the next step. |
| **Step 5. Add sample type-specific buffer, samples, standards and blanks and incubate** | **A.** This kit is supplied with 10X Universal Assay Buffer. Prepare a 1X working concentration of Universal Assay Buffer by mixing 10 mL of the 10X Universal Assay Buffer with 90 mL dd$H_2O$.<br>**B.** Add 25 µL of Universal Assay Buffer (1X) to each well for serum and plasma followed by 25 µL of standards or samples into dedicated wells. For cell culture supernatant samples, add 50 µL standards or samples into dedicated wells.<br>**C.** For wells designated as blanks, add an additional 25 µL of Universal Assay Buffer for serum or plasma samples. For cell culture supernatant samples, add 50 µL of cell culture medium.<br>**D.** Seal the plate with the provided Plate Seal. Cover the plate with the Black Microplate Lid and shake at 500 rpm for 60 min at room temperature (RT). |
| **Step 6. Wash the 96-Well Plate** | Wash plate for a total of three washes using "Step 4. Wash Antibody Magnetic Beads". |

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 7. Prepare 1X Detection Antibody Mixture</td><td>A. Detection antibody is provided at 50X concentration. Add 60 µL of each detection antibody concentrate to the mixing bottle and bring volume of the mixing bottle to a total of 3 mL using detection antibody diluent.Table below is an example for 48 and 96-wells.<br><table><tr><th></th><th>48-wells</th><th>96-wells</th></tr><tr><td>Detection Antibody</td><td>30 µL</td><td>60 µL</td></tr><tr><td>Detection Antibody Diluent</td><td>1470 µL</td><td>2940 µL</td></tr><tr><td>Total</td><td>1500 µL</td><td>3000 µL</td></tr></table></td></tr>
<tr><td>Step 8. Add Detection Antibody Mixture and incubate</td><td>A. Add 25 µL of Detection Antibody Mixture (1X) to each well.<br>B. Seal the plate with a new Plate Seal, cover plate with Black Microplate Lid and incubate 30 min on a plate shaker at RT at 500 rpm.</td></tr>
<tr><td>Step 9. Wash the 96-Well Plate</td><td>Wash plate for a total of three washes using "Step 4. Wash Antibody Magnetic Beads".</td></tr>
<tr><td>Step 10. Prepare the 96-Well Plate for Analysis on a Luminex Instrument</td><td>A. Add 120 µL of Reading Buffer into each well.<br>B. Seal the plate with a new Plate Seal, cover plate with Black Microplate Lid and incubate 5 min on a plate shaker at RT at 500 rpm.<br>C. Remove Plate Seal and run the plate on a Luminex Instrument.</td></tr>
</table>

## Setup of the Luminex Instruments

| Instrument | Sample Size | DD Gate | Timeout | Bead Event/Bead Region |
|---|---|---|---|---|
| Luminex 100/200 FLEXMAP 3D | 50 µL | 5,000 - 25,000 | 60 sec | 50-100 |
| MAGPIX | 50 µL | N/A | N/A | 50-100 |

Prior to running the assay, ensure that the probe height has been calibrated with 96-Well Flat Bottom Plate supplied with the kit. Failure to adjust the probe height can cause damage to the instrument or low bead count. The Luminex system allows for calibration of Low and High RP1 target values. We recommend RP1 Low target value settings for ProcartaPlex Immunoassays. Please refer to the Certificate of Analysis provided with the kit for bead region and analyte associations when entering the information into the Luminex Acquisition Software.

**NOTE: If there is a malfunction of the Luminex Instrument or software during the run, the 96-Well Flat Bottom Plate can be re-read. Remove the 96-Well Flat Bottom Plate from the instrument, insert the 96-Well Flat Bottom Plate into the Hand-Held Magnetic Plate Washer, wait 2 min, then remove the buffer in the wells by quickly inverting the 96-Well Flat Bottom Plate over a sink or waste container. Blot the assembly onto several layers of paper towels to remove any residual solution. Resuspend the beads in 120 µL of Reading Buffer, remove from the Hand-Held Magnetic Plate Washer, seal the 96-Well Flat Bottom Plate with a new Plate Seal and Lid and shake at 500 rpm for 5 min at room temperature. The assayed samples may take longer to read since there will be less beads in the well.**

## Analyzing Results

The concentration of the samples can be calculated by plotting the expected concentration of the standards against the MFI generated by each standard. A 4PL or 5PL algorithm is recommended for the best curve fit. Analyze the assayed samples according to the operation manual for the Luminex Instrument (e.g MAGPIX®, Luminex® 100/200™, FLEXMAP 3D®). We offer a free and robust analysis software package for data analysis. ProcartaPlex Analyst 1.0 can be download at: **www.ebioscience.com/resources/procartaplex-analyst-1.0-software.htm**

# Troubleshooting

| Observation | Probable Cause | Recommend Solution |
|---|---|---|
| Low Flow Rate | Samples/beads are stuck in flow cell | Remove the 96-Well Plate and perform a wash and rinse cycle. |
| High CVs | Samples and antigen standards not stored on ice | Prepare the samples and standards on ice before setting up the assay. |
| | Contamination from re-using the Plate Seal | Use a new Plate Seal for each incubation step. |
| | Incomplete washing | After adding the standards and samples, it is very important that any excess standards are removed during the wash step. |
| | Contamination from contents from adjacent wells | Avoid splashing the Wash Buffer during wash steps into adjacent wells. |
| | Poor pipetting techniques | Use a multichannel pipettor and careful pipette techniques. Avoid touching pipette tips to sides of the wells when adding Wash Buffer. |
| Limited dynamic range for BioPlex software users | Instrument calibrated at high PMT settings | Calibrate the instrument using the CAL2 Low RP1 target value. |
| Low bead count | Volume of bead solution is too low | Add 120 µL Reading Buffer into each well and shake at 500 rpm for 5 min at room temperature to resuspend beads prior to reading on the Luminex Instrument. |
| | High bead aggregation | Vortex the bead suspension well before using in the assay and ensure that the beads are properly mixed during the incubation steps. |
| | Dyes contained in the beads are photo-bleached from overexposure to light | Store bead solution and the 96-well plate in the dark. |
| | Samples causing the instrument to clog | Remove the 96 Well Flat Bottom Plate and perform a wash and rinse to the instrument. Rerun the assay with further dilution of samples |
| | Probe height is incorrect | Refer to the Luminex Manual for proper adjustment of the needle height. |
| | Instrument needle is partially clogged | Replace or clean needle according to the manufacturer's recommendations. |
| | Beads stuck to the bottom of the plate | Confirm that the plate shaker is set to 500 rpm and shaking for at least 5 min before reading. |
| | Air bubble in the sample loop | Refer to the Luminex manual for proper removal of the air bubble. |
| Low signal or sensitivity | Standards not reconstituted and diluted correctly | Prepare fresh antigen standards following the instructions in the Preparing Antigen Standards section. |
| Poor recovery | Did not use appropriate cell culture media to prepare the standards | Use the same cell culture media that is used to culture the cells. |
| | Samples and antigen standards were not stored on ice | Prepare the samples and standards on ice before setting up the assay. |

# Recommended and Blank Plate Layout

| Standards | | Samples | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Standard 1 | Standard 1 | 1 | 1 | 9 | 9 | 17 | 17 | 25 | 25 | 33 | 33 |
| Standard 2 | Standard 2 | 2 | 2 | 10 | 10 | 18 | 18 | 26 | 26 | 34 | 34 |
| Standard 3 | Standard 3 | 3 | 3 | 11 | 11 | 19 | 19 | 27 | 27 | 35 | 35 |
| Standard 4 | Standard 4 | 4 | 4 | 12 | 12 | 20 | 20 | 28 | 28 | 36 | 36 |
| Standard 5 | Standard 5 | 5 | 5 | 13 | 13 | 21 | 21 | 29 | 29 | 37 | 37 |
| Standard 6 | Standard 6 | 6 | 6 | 14 | 14 | 22 | 22 | 30 | 30 | 38 | 38 |
| Standard 7 | Standard 7 | 7 | 7 | 15 | 15 | 23 | 23 | 31 | 31 | 39 | 39 |
| Blank | Blank | 8 | 8 | 16 | 16 | 24 | 24 | 32 | 32 | 40 | 40 |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | | | | | | | | | | | | |
| **B** | | | | | | | | | | | | |
| **C** | | | | | | | | | | | | |
| **D** | | | | | | | | | | | | |
| **E** | | | | | | | | | | | | |
| **F** | | | | | | | | | | | | |
| **G** | | | | | | | | | | | | |
| **H** | | | | | | | | | | | | |